



**Full Length Article**

## Induction and Proliferation of *Moringa oleifera* Somatic Embryo Callus using Solid Liquid and Temporary Immersion System

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### Abstract

*Moringa oleifera* Lam., a promising functional food, can be propagated rapidly through somatic embryogenesis technique. This method has advantages, such as rapid seed generation, absence of a dormant phase, and bipolar cotyledons. The induction of *M. oleifera* callus involves plant growth regulators, the type of explants, and the media used. In this study, we investigated the effects of 0.0, 0.5, 1.0 and 2.0 mg/L of BAP and picloram on inducing embryonic callus in *M. oleifera* plants. Additionally, we examined the impact of amino acids such as proline, glutamine, and cysteine on the proliferation stage of the embryonic callus. The results showed that 1 mg/L BAP and picloram significantly enhanced callus induction, as indicated by increased callus diameter, weight, and volume. The growth of *M. oleifera* callus was also influenced by the concentration of amino acids. The optimal proliferation callus was achieved using 200 mg/L of proline in Driver and Kuniyuki Walnut (DKW) medium with the Temporary Immersion System (TIS). Furthermore, embryogenic callus was generated using 200 mg/L proline, 50 mg/L glutamine, and 50 and 100 mg/L cysteine. The addition of 200 mg/L proline to DKW yielded the best results in TIS.

**Keywords:** *Moringa oleifera*; Callus; Somatic embryo; Induction; Proliferation

### Introduction

*Moringa oleifera* Lam. has received attention because of its potential as an alternative functional food for preventing and reducing nutritional deficiencies as well as its health advantages. This species originates in the sub-Himalayan region of southwestern India (Oyeyinka and Oyeyinka 2018). *Moringa* plants are currently cultivated in many tropical and subtropical regions worldwide. Indonesia has also supported this plant through its sustainable cultivation and domestication (Toyosi *et al.* 2021).

Leaves and seeds of *M. oleifera* contain a variety of essential chemical components, including fatty acids, tocopherol, carotene, and phenolics. *M. oleifera* seeds and fruits can be used to prevent colon cancer and digestive issues. Additionally, the moringa flowers have the potential to impart color and flavor to culinary preparations (Ziani *et al.* 2019; Padayachee and Bajinath 2020; Fernandes *et al.* 2021).

The conventional propagation of *M. oleifera* commonly uses cuttings (vegetatively) and seeds (generatively). This method has several obstacles, such as susceptibility to pests and pathogens. To address this issue, one potential method for the mass propagation of *M. oleifera* is to induce somatic embryos. This method has many advantages, such as the ability to produce a lot of seeds, the lack of a dormant phase, the presence of both shoot tips and supporting roots, and the ability to use the material as an explant for genetic modification to improve plant traits (Jha *et al.* 2007). Somatic embryogenesis is a method of plant multiplication that allows the production of a high quantity of plants throughout the year. Additionally, it has the potential to be a valuable tool for enhancing the genetic characteristics of any plant species due to its single-cell origin (Jha *et al.* 2007).

*In vitro* plant propagation often employs many types of media, including solid, semisolid, and liquid media. The

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differences in media types were determined by the species, explant type and plant age. The Temporary Immersion System (TIS) was specifically designed for large-scale *In vitro* plant propagation. TIS is more efficient in promoting plant growth because of the uniform conditions provided by the chambers and media. Using TIS can improve the maturity and consistency of the somatic embryo callus (Ernayunita and Taryono 2020).

The aims of this study were to examine the effect of BAP and picloram on the production of the embryonic somatic callus of *M. oleifera* and to optimize the callus proliferation stage by adding amino acids including proline, glutamine, and cysteine in solid, liquid, and TIS media.

## Materials and Methods

### Embryogenic callus induction

The explants utilized were young meristematic *M. oleifera* shoots from seed sprouts that had been sterilized two weeks after culture. The basal medium used was Driver and Kuniyuki Walnut (DKW) medium (Driver and Kuniyuki 1984) medium supplemented with 0.0, 0.5, 1.0 and 2.0 mg/L BAP in combination with picloram, according to the treatment tested. Plant tissue culture equipment was used, and the experiment was carried out using a completely randomized factorial design. The tested factors were plant growth regulators (control, BAP, and picloram) and concentrations of 0.0, 0.5, 1.0 and 2.0 mg/L. The number of replicates was 20, with a total of 320 experimental units. The observed variables included the percentage of callus formation, callus diameter, percentage of embryogenic callus, color, and condition of explants at 0–6 weeks after culture (WAC), as well as the morphology and histology of the somatic embryonic callus at 6 WAC.

### Proliferation of somatic embryos callus

In this experiment, callus of *M. oleifera* at 0.025–0.030 g fresh weight and 0.4–0.5 cm in diameter were cultured on Driver and Kuniyuki Walnut (DKW) (Driver and Kuniyuki 1984) and solidified with 3 g/L of Gelzan (TM Caissonlabs) supplemented with amino acids (proline, glutamine, and cysteine) at 0, 20, 100, 150 and 200 mg/L. The medium pH was adjusted to 5.8 prior to sterilization using an autoclave at 15 psi at 121°C for 20 min. The cultures were incubated at  $24 \pm 2^\circ\text{C}$  with continuous light at an intensity of  $4.29\text{--}7.14 \mu\text{mol/m}^2/\text{s}$ . The experimental design was a completely randomized factorial design, examining the effects of different amino acids (proline, glutamine, and cysteine) at concentrations of 0, 20, 100, 150, and 200 mg/L. The study consisted of 16 replicates, and 240 clumps were used as experimental units. The factors measured included callus diameter, callus color, callus condition or structure, number of globular embryos, embryo weight, callus volume, tissue analysis, and

stereomicroscope observations conducted between 0 and 6 WAC. The best concentration of amino acids was used in the solid media employed for liquid treatment and the TIS. These experiments involved a completely randomized design to observe the weight and volume of embryo callus at 6 WAC.

### Data analysis

Data were analyzed using analysis of variance (ANOVA) to determine the significant levels of differences in responses between treatments. Responses that differed significantly were compared using Duncan's multiple range test (DMRT) at  $\alpha=5\%$  probability level using DSAASTAT V.1.1 (open-source software).

## Results

### Embryogenic callus induction

To investigate the effect of BAP and picloram on embryogenic callus induction in DKW medium, the callus diameter, callus weight and callus volume were analyzed using analysis of variance (ANOVA) at 6 WAC, as data displayed on Table 1. The analytical data indicated that the addition of BAP did not have a significant impact on callus diameter or callus weight, but it did have a significant effect on callus volume. On the other hand, the application of picloram had a notable impact on the callus diameter but did not affect its weight or volume. An interaction occurs between BAP and picloram, which affects callus diameter. However, analysis of the two factors revealed no interaction between callus weight and callus volume (Table 1).

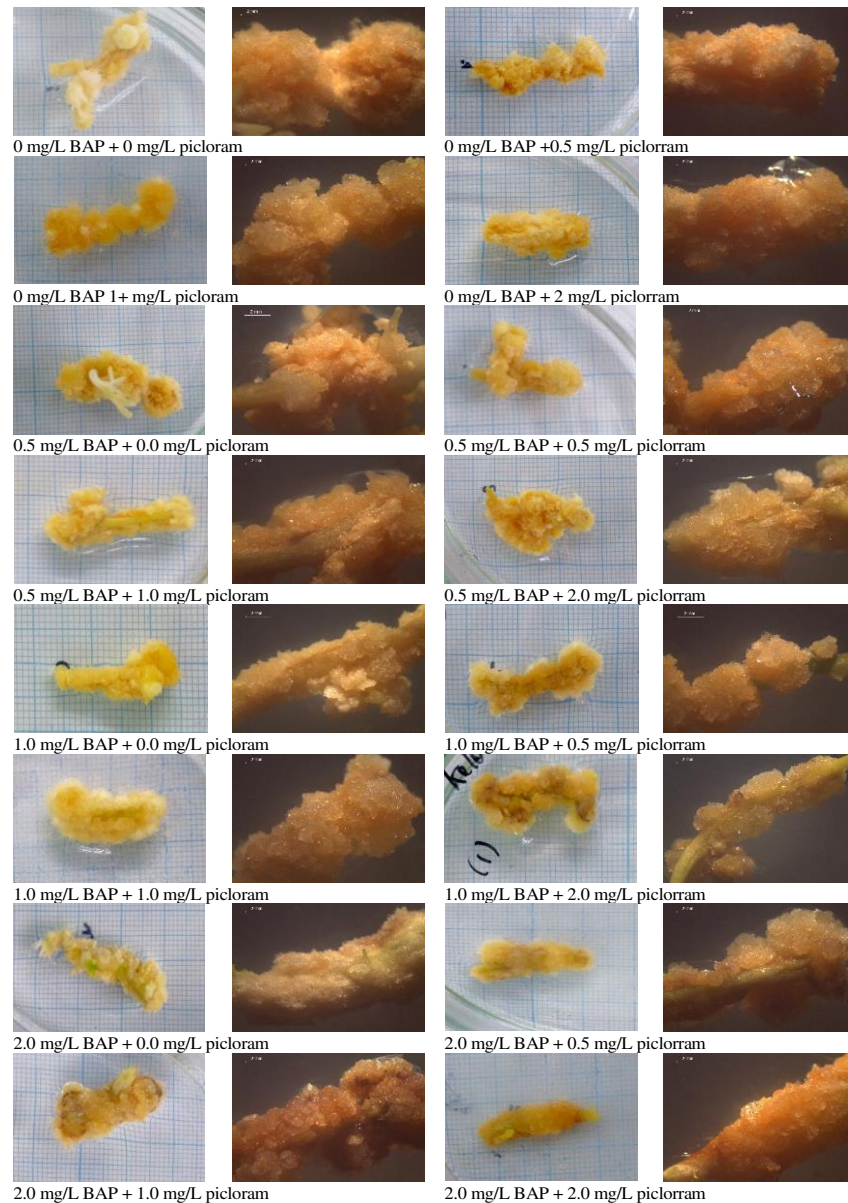
The mean value of diameter, weight, and volume of calluses from *M. oleifera* that were cultured on DKW medium with different concentrations of BAP and picloram (0.0, 0.5, 1.0 and 2.0 mg/L) at 6 WAC is displayed in Table 2. Treatment with 1 mg/L BAP and 1 mg/L picloram resulted in large callus diameters, as did the callus weight and callus volume. However, these treatments did not significantly differ from the others, except for the combination of 1 mg/L BAP with 0 and 2 mg/L picloram, and the treatment with 2 mg/L BAP with 2 mg/L picloram (Table 2).

The performance of *M. oleifera* callus cultured with DKW medium and supplemented with different concentrations of BAP and picloram (0.0, 0.5, 1.0 and 2.0 mg/L) at 6 WAC is depicted in Fig. 1. The *M. oleifera* explants were able to form callus in all treatments tested. Without the addition of BAP and picloram, the treatment produced a callus that was not crumbly, some of which browned and did not proliferate optimally until six weeks after culture. The combination of 1 mg/L BAP and 1 mg/L picloram produced a crumbly callus, with all parts of the explant forming a perfect callus. In this treatment, callus growth was better than in the other treatments, with a relatively higher callus diameter compared to the control. In

**Table 1:** Presents the results of an analysis of variance (ANOVA) conducted on the DKW medium, specifically examining the effects of adding BAP paired with picloram on callus diameter, callus weight, and callus volume at 6 WAC.

Variable	F value and significance			CV (%)
	BAP	Picloram	BAP × picloram	
Callus diameter	0.66 <sup>ns</sup>	8.23 <sup>**</sup>	2.07 <sup>*</sup>	24.62
Callus weight	2.27 <sup>ns</sup>	1.78 <sup>ns</sup>	2.06 <sup>ns</sup>	45.80
Callus volume	7.21 <sup>**</sup>	1.73 <sup>ns</sup>	2.07 <sup>ns</sup>	40.34

Significant at: \* P&lt;0.05, \*\* P&lt;0.01 and ns non-significant

**Fig. 1:** The performance of *M. oleifera* callus cultured with DKW medium and supplemented with 0.0, 0.5, 1.0 and 2.0 mg/L of BAP in combination with picloram at 6 WAC

the treatment with 2 mg/L BAP combined with 2 mg/L picloram, only a small amount of callus was formed, with slow callus growth. Some calluses even died and browned at 6 WAC. At BAP concentrations greater than 1 mg/L, *M. oleifera* callus formation and proliferation decreased (Fig. 1).

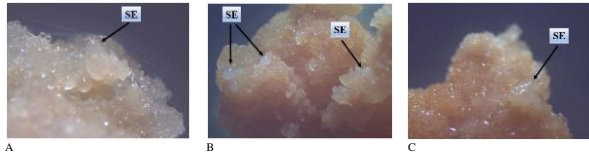
### Proliferation of somatic embryos callus

Table 3 presents the analysis of variance (ANOVA) on the variables of callus diameter, callus weight, and callus volume of *M. oleifera* grown on DKW media supplemented with 0.0,

**Table 2:** Mean value of callus diameter, callus weight, and callus volume of *M. oleifera* that were cultured on DKW medium supplemented with 0.0, 0.5, 1.0 and 2.0 mg/L of BAP and picloram at 6 WAC

BAP (mg/L)	Picloram (mg/L)	Callus diameter (mm)	Callus weight (mg)	Callus volume (mL)
0.0	0.0	19.33 ± 1.23 <sup>abc</sup>	585.45 ± 52.61 <sup>ab</sup>	0.95 ± 0.08 <sup>a</sup>
	0.5	14.17 ± 1.38 <sup>d</sup>	338.68 ± 19.80 <sup>b</sup>	0.66 ± 0.04 <sup>abc</sup>
	1.0	21.17 ± 0.71 <sup>ab</sup>	503.73 ± 18.32 <sup>ab</sup>	0.68 ± 0.04 <sup>abc</sup>
	2.0	19.25 ± 0.77 <sup>abc</sup>	610.95 ± 33.15 <sup>ab</sup>	0.85 ± 0.03 <sup>a</sup>
0.5	0.0	17.67 ± 1.19 <sup>abcd</sup>	359.23 ± 48.66 <sup>b</sup>	0.56 ± 0.08 <sup>abc</sup>
	0.5	18.17 ± 1.67 <sup>abcd</sup>	585.40 ± 113.74 <sup>ab</sup>	0.74 ± 0.12 <sup>ab</sup>
	1.0	21.67 ± 0.83 <sup>a</sup>	576.33 ± 46.04 <sup>ab</sup>	0.88 ± 0.04 <sup>a</sup>
	2.0	18.83 ± 1.16 <sup>abc</sup>	501.18 ± 16.17 <sup>ab</sup>	0.60 ± 0.03 <sup>abc</sup>
1.0	0.0	16.83 ± 1.63 <sup>bcd</sup>	289.65 ± 15.98 <sup>b</sup>	0.39 ± 0.02 <sup>bc</sup>
	0.5	19.08 ± 1.03 <sup>abc</sup>	628.90 ± 70.43 <sup>ab</sup>	0.74 ± 0.08 <sup>ab</sup>
	1.0	<b>21.33 ± 1.23<sup>a</sup></b>	<b>756.15 ± 54.36<sup>a</sup></b>	<b>0.90 ± 0.05<sup>a</sup></b>
	2.0	14.33 ± 1.69 <sup>d</sup>	311.95 ± 41.42 <sup>b</sup>	0.38 ± 0.05 <sup>bc</sup>
2.0	0.0	18.25 ± 1.29 <sup>abcd</sup>	303.88 ± 15.93 <sup>b</sup>	0.36 ± 0.01 <sup>bc</sup>
	0.5	17.83 ± 1.82 <sup>abcd</sup>	491.55 ± 94.47 <sup>ab</sup>	0.58 ± 0.10 <sup>abc</sup>
	1.0	20.33 ± 1.34 <sup>ab</sup>	303.83 ± 58.65 <sup>b</sup>	0.34 ± 0.06 <sup>bc</sup>
	2.0	15.83 ± 1.33 <sup>cd</sup>	277.23 ± 19.36 <sup>b</sup>	0.30 ± 0.02 <sup>c</sup>

Significant at: \* P<0.05, \*\* P<0.01 and ns non-significant. Numbers followed by the same letter in the same column are not significantly different based on Duncan's multiple range test at  $\alpha = 5\%$ . Presented values are means  $\pm$  standard error (SE) of 20 replicates

**Fig. 2:** Proliferation of *M. oleifera*'s callus using the TIS**Fig. 3:** Microscopic observations of *M. oleifera* embryogenic callus on DKW medium supplemented with 200 mg/L of proline (A), 50 mg/L of glutamine (B) and 50 mg/L of cysteine (C).

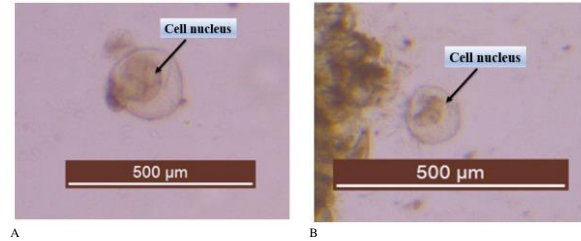
50, 100, 150 and 200 mg/L of amino acids (proline, glutamine, and cysteine). The analysis revealed that amino acid type had a statistically significant impact on the callus diameter variable but did not have a significant influence on the callus weight or callus volume variables. Conversely, the concentration factors significantly influenced all examined variables. There is an interaction between the amino acid type and the concentration on the variables i.e., callus diameter, callus weight, and callus volume (Table 3).

Table 4 displays the mean values of callus diameter, callus weight, and callus volume of *M. oleifera* cultivated on DKW medium containing 0.0, 50, 100, 150 and 200 mg/L of amino acids (proline, glutamine, and cysteine, respectively) at 6 WAC. The proline and cysteine treatments at 200 mg/L showed high callus diameter, weight, and volume, but were

**Table 3:** Analysis of variance (ANOVA) on the variables of callus diameter, callus weight, and callus volume of *M. oleifera* grown on DKW media supplemented with 0.0, 50, 100, 150 and 200 mg/L of amino acids at 6 WAC

Variable	F value and significance			CV × (%)
	Amino acids	Concentration	Amino acids concentration	
Callus diameter	3.86*	3.66**	2.18*	37.03
Callus weight	1.65 <sup>ns</sup>	3.16*	2.83**	75.83
Callus volume	2.69 <sup>ns</sup>	3.20*	2.54*	77.71

Significant at: \* P<0.05, \*\* P<0.01 and ns non-significant

**Fig. 4:** *M. oleifera* embryogenic callus on DKW medium supplemented with 150 mg/L cysteine (A) and 50 mg/L cysteine (B)

not significantly different from the other treatments.

The mean value of weight and volume of callus from *M. oleifera* cultured on DKW media supplemented with 0.0 and 200 mg/L proline using solid, liquid, and TIS at 6 WAC are presented in Table 5. The first culture of callus on solid, liquid, and TIS had an average weight ranging from 0.05–0.07 g, with 0.06–0.09 mL of volume. After culturing for 6 weeks, the weight of the callus on 0 mg/L proline on solid medium was 0.7283 and 0.8733 g on media with 200 mg/L proline. The solid media using DKW and 0.0 mg/L of proline produced 0.7667 mL of callus volume, whereas media supplemented with 200 mg/L proline had a 0.6500 mL of volume. In liquid medium, DKW with 0 mg/L proline produced calluses with varying weights and volumes, with the weight being 0.1367 g and the volume being 0.1617 mL. In contrast, in DKW supplemented with 200 mg/L proline, the weight was 0.1717 g and the volume was 0.1783 mL.

The TIS was used in this experiment to evaluate the growth of *M. oleifera* callus. The DKW and 0 mg/L proline had callus weight and volume of 1.2050 g and 1.1500 mL, respectively. The DKW supplemented with 200 mg/L proline had 1.6200 g of weight and 1.9000 mL of volume. The addition of 200 mg/L proline did not significantly enhance callus proliferation in solid or liquid medium. However, in the TIS method, the addition of 200 mg/L proline led to a considerable increase in the callus weight and volume. The most effective method for proliferating *M. oleifera* calli is through the TIS using DKW media supplemented with 200 mg/L of proline, as indicated in Table 5.

The qualitative observation data on the colour, number, and condition of *M. oleifera* callus on DKW media supplemented with 0, 50, 100, 150 and 200 mg/L of amino

**Table 4:** Presents the mean diameter, weight, and volume of the callus of *M. oleifera* in DKW media supplemented with 0.0, 50, 100, 150 and 200 mg/L of amino acids at 6 WAC

Amino acid	Concentration (mg/L)	Callus diameter (mm)	Callus weight (mg)	Callus volume (mL)
Proline	0	1.41 ± 0.15 <sup>abc</sup>	0.51 ± 0.12 <sup>abcd</sup>	0.65 ± 0.09 <sup>ab</sup>
	50	1.54 ± 0.11 <sup>ab</sup>	0.60 ± 0.12 <sup>ab</sup>	0.69 ± 0.13 <sup>a</sup>
	100	0.93 ± 0.18 <sup>cd</sup>	0.19 ± 0.07 <sup>de</sup>	0.25 ± 0.09 <sup>cd</sup>
	150	1.44 ± 0.19 <sup>abc</sup>	0.38 ± 0.09 <sup>abcde</sup>	0.38 ± 0.11 <sup>abcd</sup>
	200	<b>1.66 ± 0.13<sup>a</sup></b>	<b>0.68 ± 0.16<sup>a</sup></b>	<b>0.71 ± 0.20<sup>a</sup></b>
Glutamine	0	1.35 ± 0.19 <sup>abc</sup>	0.37 ± 0.10 <sup>abcde</sup>	0.41 ± 0.09 <sup>abcd</sup>
	50	1.05 ± 0.22 <sup>bcd</sup>	0.30 ± 0.14 <sup>bcd</sup>	0.24 ± 0.10 <sup>cd</sup>
	100	1.26 ± 0.21 <sup>abc</sup>	0.48 ± 0.14 <sup>abcd</sup>	0.54 ± 0.17 <sup>abc</sup>
	150	1.20 ± 0.12 <sup>abc</sup>	0.36 ± 0.06 <sup>abcde</sup>	0.38 ± 0.06 <sup>abcd</sup>
	200	1.23 ± 0.22 <sup>abc</sup>	0.31 ± 0.14 <sup>bcd</sup>	0.36 ± 0.17 <sup>abcd</sup>
Cysteine	0	1.36 ± 0.04 <sup>abc</sup>	0.55 ± 0.05 <sup>abc</sup>	0.56 ± 0.06 <sup>abc</sup>
	50	0.63 ± 0.09 <sup>d</sup>	0.09 ± 0.01 <sup>e</sup>	0.09 ± 0.01 <sup>d</sup>
	100	1.03 ± 0.19 <sup>bcd</sup>	0.23 ± 0.11 <sup>cde</sup>	0.27 ± 0.13 <sup>bcd</sup>
	150	1.03 ± 0.10 <sup>bcd</sup>	0.28 ± 0.06 <sup>bcd</sup>	0.28 ± 0.05 <sup>bcd</sup>
	200	1.53 ± 0.17 <sup>ab</sup>	<b>0.68 ± 0.12<sup>a</sup></b>	<b>0.71 ± 0.16<sup>a</sup></b>

Significant at: \* P<0.05, \*\* P<0.01 and ns non-significant. Numbers followed by the same letter in the same column are not significantly different based on Duncan's multiple range test at  $\alpha = 5\%$ . Presented values are means  $\pm$  standard error (SE) of 20 replicates.

**Table 5:** Mean value of callus weight and callus volume of *M. oleifera* cultured on DKW media supplemented with 0.0 and 200 mg/L proline in solid, liquid, and TIS methods

Media	Proline (mg/L)	Callus weight (mg)	Callus volume (mL)
Solid	0	0.73 ± 0.02 <sup>c</sup>	0.77 ± 0.02 <sup>c</sup>
	200	0.87 ± 0.08 <sup>c</sup>	0.65 ± 0.01 <sup>c</sup>
Liquid	0	0.14 ± 0.01 <sup>d</sup>	0.16 ± 0.16 <sup>d</sup>
	200	0.17 ± 0.01 <sup>d</sup>	0.18 ± 0.16 <sup>d</sup>
TIS	0	1.21 ± 0.14 <sup>b</sup>	1.15 ± 0.00 <sup>b</sup>
	200	<b>1.62 ± 0.05<sup>a</sup></b>	<b>1.90 ± 0.04<sup>a</sup></b>

Significant at: \* P<0.05, \*\* P<0.01 and ns non-significant. Numbers followed by the same letter in the same column are not significantly different based on Duncan's multiple range test at  $\alpha = 5\%$ . Presented values are means  $\pm$  standard error (SE) of 20 replicates

**Table 6:** Callus colour, number of calluses, and condition of *M. Oleifera* callus on DKW Media supplemented with 0.0, 50, 100, 150 and 200 mg/L of amino acids at 6 WAC

Amino acid	Concentration (mg/L)	Callus colours		Number of callus		Callus condition	
		0 WAC	6 WAC	0 WAC	6 WAC	0 WAC	6 WAC
Proline	0	white	brown	few	enormous quantity	fresh	fresh; browning
	50	white	yellow	few	enormous quantity	fresh	fresh
	100	white	brown	few	few	fresh	stuck; browning
	150	white	brown	few	enormous quantity	fresh	fresh; browning
	200	white	yellow	few	enormous quantity	fresh	fresh
Glutamine	0	white	yellow	few	enormous quantity	fresh	fresh
	50	white	brown	few	few	fresh	stuck; browning
	100	white	brown	few	enormous quantity	fresh	fresh; browning
	150	white	yellow	few	enormous quantity	fresh	fresh
	200	white	yellow	few	few	fresh	stuck
Cysteine	0	white	brown	few	enormous quantity	fresh	fresh; browning
	50	white	brown	few	few	fresh	stuck; browning
	100	white	yellow	few	few	fresh	stuck
	150	white	brown	few	lots	fresh	fresh; browning
	200	white	yellow	few	enormous quantity	fresh	fresh

acids (proline, glutamine, and cysteine) at 6 WAC are presented in Table 6. During the initial week of callus monitoring, it appeared white in colour. However, by the sixth week of culture, the callus of *M. oleifera* had undergone a colour transformation and turned yellowish. The initial count of calluses treated with 200 mg/L proline at week 0 indicated a limited number. However, by the 6th week, the callus count had significantly increased. At 6 WAC, the condition of the callus indicates that the callus

explant is still fresh (Table 6). An increase in the quantity of callus signifies proliferation of callus formation.

Microscopic observations of *M. oleifera* embryogenic callus cultured on DKW media supplemented with amino acids at 6 WAC are presented in Fig. 3. Embryogenic callus was generated on DKW medium supplemented with 200 mg/L proline, 50 mg/L glutamine, and 50 mg/L cysteine (Fig. 3). Fig. 4 depicts the analysis of *M. oleifera* callus tissue at 6 WAC using an inverted microscope. *M. oleifera*

callus, which formed embryogenic callus in the DKW media with the addition of 50 mg/L cysteine (Fig. 4A) and 150 mg/L cysteine (Fig. 4B).

## Discussion

Somatic embryos can be differentiated through direct somatic embryogenesis (DSE) or indirect somatic embryogenesis (ISE) derived from embryogenic callus cells. Both processes are influenced by internal and external factors such as the explant's characteristics, culture media composition, plant growth regulators (PGRs), temperature, and light conditions (Bogdanovic *et al.* 2021). In this study, callus induction of *M. oleifera* somatic embryos was carried out through an indirect somatic embryo process using meristematic young shoot explants, which were cultured on DKW media supplemented with BAP combined with picloram. The results demonstrated that applying 1 mg/L concentration of BAP and picloram significantly enhanced *M. oleifera* callus growth, as evidenced by the substantial increase in callus diameter, callus weight, and callus volume (Table 2). Khatri and Joshee (2024) report that callus induction was two times more efficient under Picloram as compared to 2,4-D in wheat, barley, and Triticum.

Explant of *M. oleifera* were able to form calluses in all tested treatments, including in the medium without the addition of BAP and picloram. In this control medium, the form of callus was not friable; some of them browned and did not proliferate optimally until six weeks after culture (Table 2, Fig. 1). Callus formation in *M. oleifera* explants can be caused by the indigenous auxin and cytokinin content in the explants. However, due to the low composition of auxin and cytokinin, the optimal growth of the callus was not achieved. Sharry *et al.* (2006) reported that the type and concentration of auxin and cytokinin employed are critical for the induction and formation of somatic embryos. However, in some cases, this PGR was not necessary in the development of somatic embryos.

The addition of proline can inhibit browning events and increase the proliferation of embryogenic callus, which initiates the growth of somatic embryos after being transferred to a medium without the addition of growth regulators (Takahashi and Takamizo 2013). Similar to our findings, Asad *et al.* (2009) found that the addition of amino acids at 0.25 mM cysteine to the medium increased somatic embryo growth by 94% in sugarcane plants.

Application of TIS on DKW media supplemented with 200 mg/L proline promoted more efficient multiplication of *M. oleifera* callus, as evidenced by the highest values of the callus weight and volume (Fig. 2, Table 5). The TIS method increases callus weight and volume by three times compared to solid media and ten times compared to liquid media. Soaking explants in a TIS can help callus better absorb nutrients, which can help them proliferate quickly (Tahardi and Riyadi 2016). The speed at which cells divide correlates with the weight and volume of the callus.

Embryogenic callus is characterised by a yellowish colour with a dry and friable callus texture. Globular callus is characterised by the appearance of small bumps on the surface of the callus and are milky white in colour. Several factors, including the explant used, the composition of the growth medium, growth regulators, and environmental conditions during the incubation period, can influence the success of the callus in forming embryogenic callus (Sugiharto *et al.* 2016). Fig. 4 displays clear cell nuclei that are round or oval in shape. Embryogenic callus can be characterised by the presence of tissue consisting of cells with clearly visible plasma and cell nuclei (Ardiyanti 2015).

## Conclusion

The embryogenic callus in this study was obtained with the addition of the amino acid proline at 200 mg/L, the amino acid glutamine at 50 mg/L, and the amino acid cysteine at concentrations of 50 mg/L and 150 mg/L. The best test results were obtained when the amino acid proline was added to DKW media grown in the TIS at a concentration of 200 mg/L.

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## Author Contributions

R and AFM planned the experiments, statistically analyzed the data and made the write up, AP and DE interpreted the results and provided a guidance and assistance

## Conflicts of Interest

All authors declare no conflict of interest

## Data Availability

Data presented in this study will be available on a fair request to the corresponding author

## Ethics Approval

Not applicable to this paper

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